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Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation

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SUMMARY

Postnatal development of the mammary gland begins during puberty with ductal proliferation and is completed at delivery with the appearance of secretory alveolar structures. Using endogenous milk protein genes and a WAP-lacZ reporter transgene, we show that the differentiation of alveolar cells is initiated in virgin mice in estrus in a limited number of cells. With the onset of pregnancy, the number of expressing cells and the cellular expression levels increase until full activity is reached at lactation. Milk protein genes are activated in a defined temporal sequence. WDNM1 and β-casein are expressed early in pregnancy and increase during alveolar proliferation. WAP (whey acidic protein) and α-lactalbumin are expressed later near the end of gestation, which is characterized by terminal differentiation of the mammary secretory phenotype. By in situ hybridization, we have established evidence for asynchrony in milk protein gene expression among alveolar cells showing large variations in the intensity of hybridization among adjacent cells. The asynchrony of maturation of epithelial cells within a given alveolus suggests that the genetic program leading to terminal differentiation is subject to local modulation. It is likely that these signals are manifest through various pathways including growth factors, the extracellular matrix or gene products specific to terminal differentiation such as WAP. We extended our analyses to WAP/WAP transgenic mice in which WAP is synthesized precociously and functional differentiation of alveolar cells is impaired. We found an altered expression pattern of milk protein genes, with a strong reduction of α-lactalbumin RNA. We conclude that the early production of WAP in WAP/WAP mammary glands disrupts the timing of gene activation leading to a premature termination of the differentiative program.

Key words: mammary gland, alveolar cells, cell differentiation, in situ hybridization

INTRODUCTION

The mammary gland provides an excellent system to study questions pertaining to organogenesis, cell differentiation and oncogenesis. The development of the gland is to a great extent governed by hormonal stimuli (Forsyth, 1986; Topper and Freeman, 1980). Development of the gland progresses gradually and is completed only in the mature organism. In newborn mice, the mammary anlagen consist of only a few ducts which under the influence of gonadal hormones during puberty and adolescence grow into the mammary fat pad. In virgin mice, the mammary parenchyma is composed of a highly organized system of ducts with terminal end buds which are the major sites of growth and lateral buds spaced regularly along the ducts. With each estrous cycle, the lateral buds differentiate. They subdivide progressively thus giving rise to small alveolar buds (Daniel and Silberstein, 1987; Russo et al., 1989). Further development and differentiation of the gland takes place during pregnancy and lactation. Between day 8 and 10 of gestation, the mammary alveoli start to form larger alveolar lobules. A rapid increase in the number and size of alveoli occurs during the second half of pregnancy resulting in the development of fully differentiated secretory lobules (Pitelka et al., 1973).

These morphogenetic events are accompanied by cellular differentiation processes leading to the development of secretory epithelial cells which are capable of synthesizing and secreting milk proteins. Low levels of milk protein genes are expressed already in virgin mice, but their synthesis increases dramatically during pregnancy following a characteristic time course: β-casein is seen earlier than the whey acidic protein (WAP) and α-lactalbumin is found only toward the end of pregnancy. Recently, WDNM1 has been identified as a milk protein gene whose expression also increases strongly during pregnancy (Morrison and Leder, 1994). Maximal expression levels of the milk protein genes are reached only during lactation.

Although lactogenic hormones are responsible for the overall induction of milk protein gene expression, they do not seem sufficient to explain the complex temporal regulation observed in the developing gland during pregnancy. Studies employing cell and organ culture as well as transgenic animals

have provided information about some of the regulatory elements involved in mammary-specific expression (Burdon et al., 1991a; Pittius et al., 1988; Shamay et al., 1992). Response elements for steroid hormones are present in the promoters of β-casein and WAP (Li and Rosen, 1994, 1995). Stat 5, a mammary gland factor involved in mediating the prolactin response has been identified and shown to bind to a specific region of the *β-casein* (Wakao et al., 1994) and *WAP* gene promoter (Li and Rosen, 1994, 1995). Interestingly, differences in the regulation between milk protein genes have been noted. Whereas the 'early' casein genes rely for their expression to a large degree on prolactin and to a lesser extent on glucocorticoids, the role of the two hormones is reversed for the 'late' *WAP* gene (Burdon et al., 1991a; Pittius et al., 1988; Shamay et al., 1992). Furthermore, the requirements for expression of β-casein and WAP in mammary cell culture have been found to be different. While β-casein is expressed by dissociated mammary epithelial cells embedded into extracellular matrix (Streuli et al., 1991), cell-to-cell contact and formation of a closed lumen are required for expression of WAP (Chen and Bissell, 1989). This strongly suggests that in addition to the lactogenic hormones, local growth factors, extracellular matrix components and a proper three-dimensional structure of the epithelium are involved in their transcriptional control (Lin and Bissell, 1993). Mammary tissue from embryos and immature virgins can be induced to express milk protein genes and to synthesize the corresponding proteins, such as β-casein and WAP, when cultured in the presence of lactogenic hormones (Pittius et al., 1988; Shamay et al., 1992). This demonstrates that alveolar cells in non-primed virgins have the capability to undergo functional differentiation in response to lactogenic hormones.

Based on the ultrastructural appearance in mid-pregnancy, it has been suggested that alveolar development is not synchronized (Mills and Topper, 1970). By morphological and immunological criteria, such as the binding of antibodies and lectins, mammary alveolar cells within individual alveoli appeared homogeneous (Ormerod and Rudland, 1984; Rudland and Hughes, 1989). However, differential keratin expression was seen among lobular alveolar cells and was attributed to the presence of dispersed latent mammogenic stem cells (Smith et al., 1990). In situ hybridization studies with ^{35}S-labeled probes for α-lactalbumin, α-S1-casein and lactoferrin have revealed large-scale regional differences throughout the mammary glands of sheep and cattle (Molenaar et al., 1992) but, due to the lower resolution of this technique, it was not possible to identify intra-alveolar differences. In another study, fluorescently labeled oligonucleotides were used to colocalize transcripts of different milk protein genes in the mammary glands of the tammar wallaby at late lactation (Joseph and Collet, 1994). At this stage, all epithelial cells in a given alveolus synthesize all milk proteins simultaneously.

In an attempt to understand the complex regulation of mammary cell differentiation, we studied alveolar cell differentiation in normal mice and in transgenic mice, which cannot develop a functional mammary gland because of the aberrant expression of a developmental protein (Burdon et al., 1991b). We performed a detailed study of the expression pattern of a mammary-gland-specific reporter gene and four major milk protein genes during pregnancy. We employed in situ hybridization utilizing non-radioactive detection methods to analyze gene transcription on a cellular level. Our results showed activation of milk protein genes in virgin animals during the estrous cycle in a small number of mammary cells. The development of fully differentiated secretory epithelial cells required additional stimuli arising during pregnancy. Perturbation of the temporal expression of a milk protein gene in transgenic mice inhibited terminal differentiation.

MATERIALS AND METHODS

Animals

C57BL/6 females were used for these studies. To obtain dated pregnancies 4- to 6-week-old virgins were superovulated and mated. The day after copulation was counted as day 0 of pregnancy. The transgenic mice carrying a mouse *WAP* transgene have been described earlier (Burdon et al., 1991b). The *WAP-lacZ* transgene was generated by ligating a 4.2 kb fragment spanning the mouse *WAP* gene promoter from −4200 to +24 to the bacterial *β-galactosidase* gene (Pharmacia). This promoter has been shown to target transcription exclusively to mammary tissue (Paleyanda et al., 1994) Transgenic mice were generated that carried approximately 2 to 10 copies of the transgene. Two lines were obtained that expressed the transgene and one was analyzed in detail.

X-Gal staining

Tissues were fixed for 2 hours in 2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP40 in PBS, rinsed in PBS and washed in X-gal staining buffer (30 mM $K_3Fe(CN)_6$, 30 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP40, in PBS) for 2 hours. The wash buffer was replaced with staining buffer containing 1 mg/ml X-gal. After sufficient color development, the tissues were transferred to 70% ethanol and processed for paraffin sectioning according to standard procedures. 5 μm sections were prepared and stained with nuclear fast red.

In situ hybridization

Mammary gland tissue was fixed in 4% paraformaldehyde in PBS and embedded in paraffin by standard methods. 5 μm sections were mounted on silan-coated slides and used for in situ hybridization. Sections were deparaffinized, treated with 2 μg/ml proteinase K for 10 minutes at room temperature, postfixed in 4% paraformaldehyde and acetylated. cRNA probes were hybridized overnight at 50°C in 50% formamide, 5× SSC, 0.1 M sodium phosphate buffer pH 7, 1× Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA and 100 μg/ml yeast tRNA. Washes were performed at 65°C in 50% formamide/2× SSC (2 times 30 minutes) and 25% formamide/1× SSC/1× PBS (2 times 30 minutes). After treating with 5 μg/ml RNAse A (Boehringer) for 10 minutes at 37°C the sections were subject to color development using the Boehringer Genius 3 kit according to the manufacturer's recommendations.

Preparation of cRNA probes

Digoxigenin-labeled probes were prepared from appropriately linearized plasmids using the Boehringer Genius 4 kit. A 250 bp cDNA sequence was used for β-casein, a 450 bp cDNA sequence for WAP, a 450 bp cDNA sequence for α-lactalbumin, and a 450 bp cDNA for WDNM1. The cDNA sequences spanned protein coding regions, and they were inserted into pBS (Stratagene, La Jolla).

Immunohistochemistry

Paraffin sections were prepared as described above. Anti-WAP antibodies (Shamay et al., 1991) were diluted 1:500 and applied overnight at 4°C. Biotinylated secondary antibodies were detected with a Vectastain ABC kit.

Extraction of RNA and northern blot hybridization

RNA was prepared according to Chomczynski and Sacchi (1987). Total RNA was separated on 1.2% formaldehyde-agarose gels, transferred to Nylon membranes (GeneScreen Plus) and hybridized. ^{32}P-labeled probes (see above) were endlabeled (oligonucleotides) or labeled by random priming (cDNAs) and used in the following order: transgene-specific oligonucleotide (Burdon et al., 1991a), α-lactalbumin, WDNM1, WAP, β-casein, 18S rRNA oligonucleotide. Hybridized probes were removed by dipping the filter in boiling water for 3 to 5 minutes. Removal of probes was checked by exposing filters overnight between hybridizations.

RESULTS

To understand the differentiation of mammary alveolar cells during puberty, pregnancy and lactation, we have analyzed the expression patterns of a mammary-specific transgene and of endogenous milk protein genes in normal mice, and in transgenic mice carrying a milk protein transgene whose expression results in aberrant mammary development.

Activity of a *WAP-lacZ* transgene during mammary gland development

We have generated transgenic mice that carry a hybrid gene consisting of 4.2 kb of the *WAP* gene promoter and the bacterial *lacZ* gene. This permitted gene expression studies with high sensitivity and single cell resolution. Expression of the transgene was detected in mammary alveolar and ductal cells of virgin mice during estrus (Fig. 1A). The activity per expressing cell was less than 10% of that found during lactation (see legend of Fig. 1). Transgene expression was transient, as we did not find β-galactosidase activity in mammary glands of transgenic mice in diestrus (Fig. 1C). No β-galactosidase activity was detected in ductal and alveolar mammary cells of non-transgenic mice in estrus (Fig. 1B).

Both the number of β-galactosidase-positive cells and the level of transgene expression increased during pregnancy, and on day 15 of pregnancy substantial expression was seen in a large number of alveolar cells (Fig. 1D). The β-galactosidase staining was preferentially observed in those cells that had a more differentiated appearance based on the presence of vacuoles. On day 18 of pregnancy, the majority of alveolar

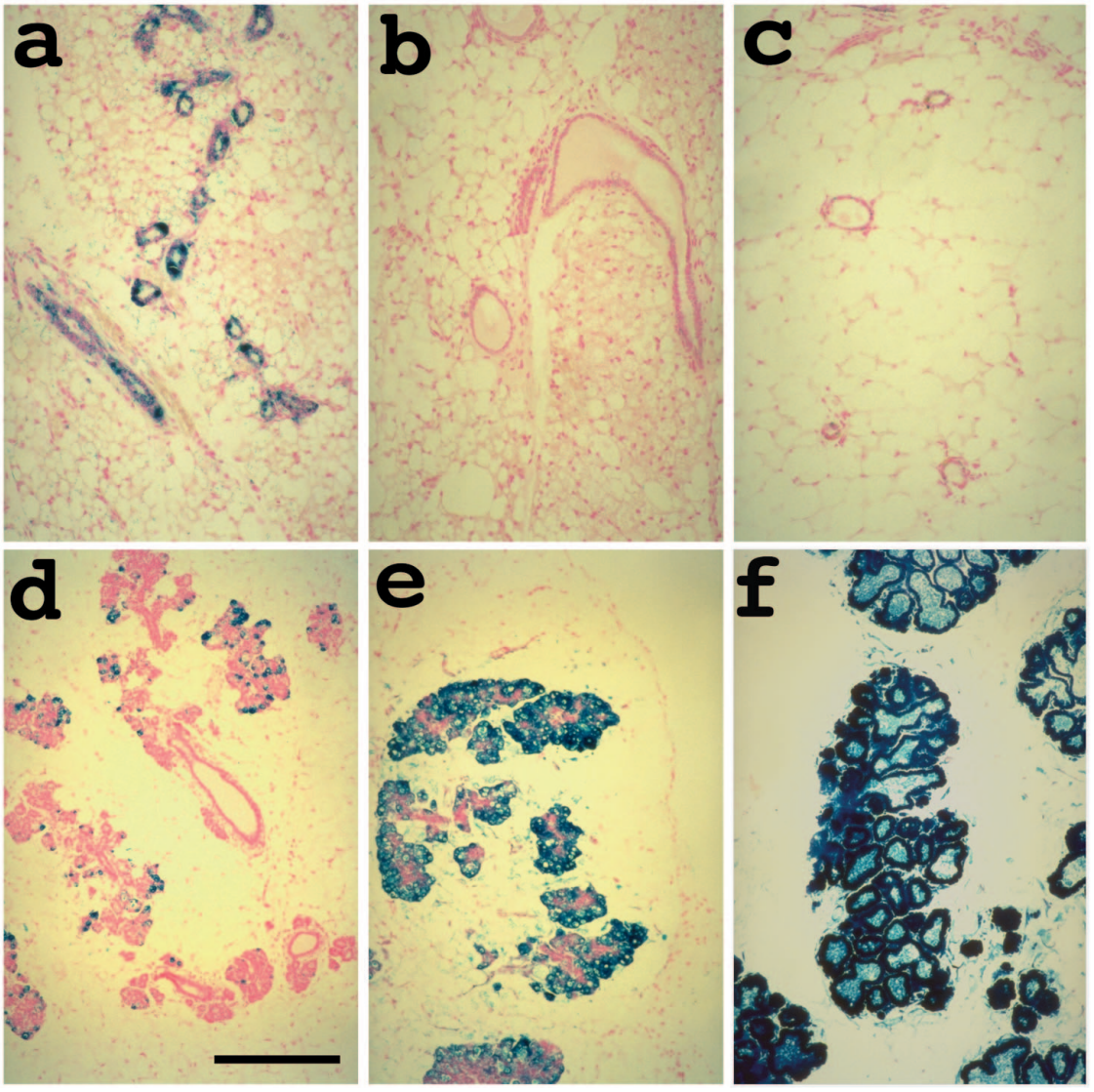
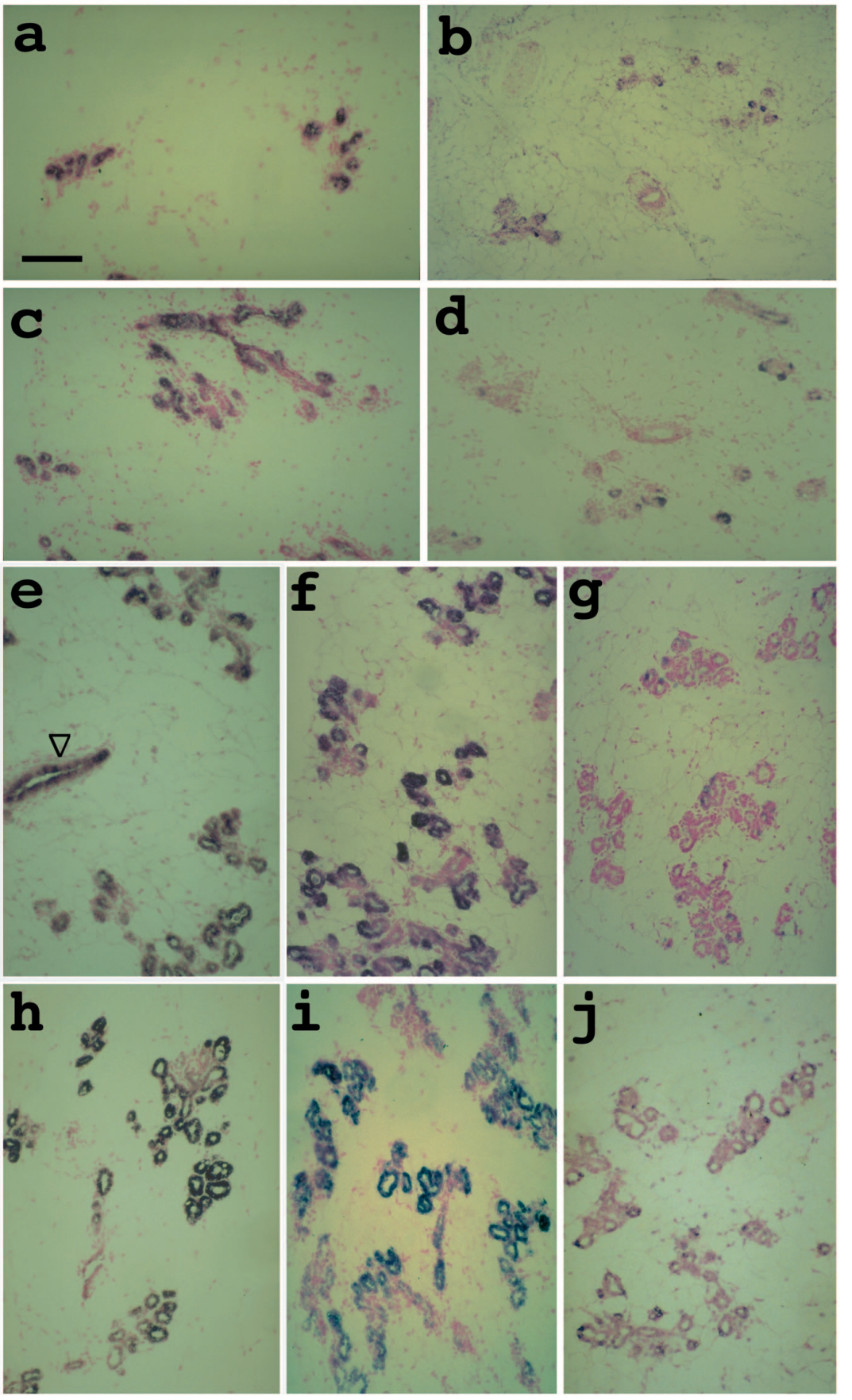


Fig. 1. Expression of a *WAP-lacZ* transgene during mammary gland development. Tissues were fixed, stained for β-galactosidase, embedded and sectioned. (a) Irregular expression is seen in mature virgins (four months of age) during estrus. (b) No staining for β-galactosidase is present in non-transgenic mature virgins of the same age during estrus. (c) β-galactosidase expression is absent in four month old transgenic virgins in diestrus. (d) Expression of the reporter transgene on day 15 of pregnancy shows irregular pattern. (e) Increased expression of the reporter transgene is seen on day 18 of pregnancy. (f) On day 3 of lactation high but variable levels of β-galactosidase are detected. Duration of the enzymatic reaction was 24 hours in a-d, in e it was 12 hours, and 4 hours in f. Bar, 100 μm (a,b,c); 200 μm (d,e,f).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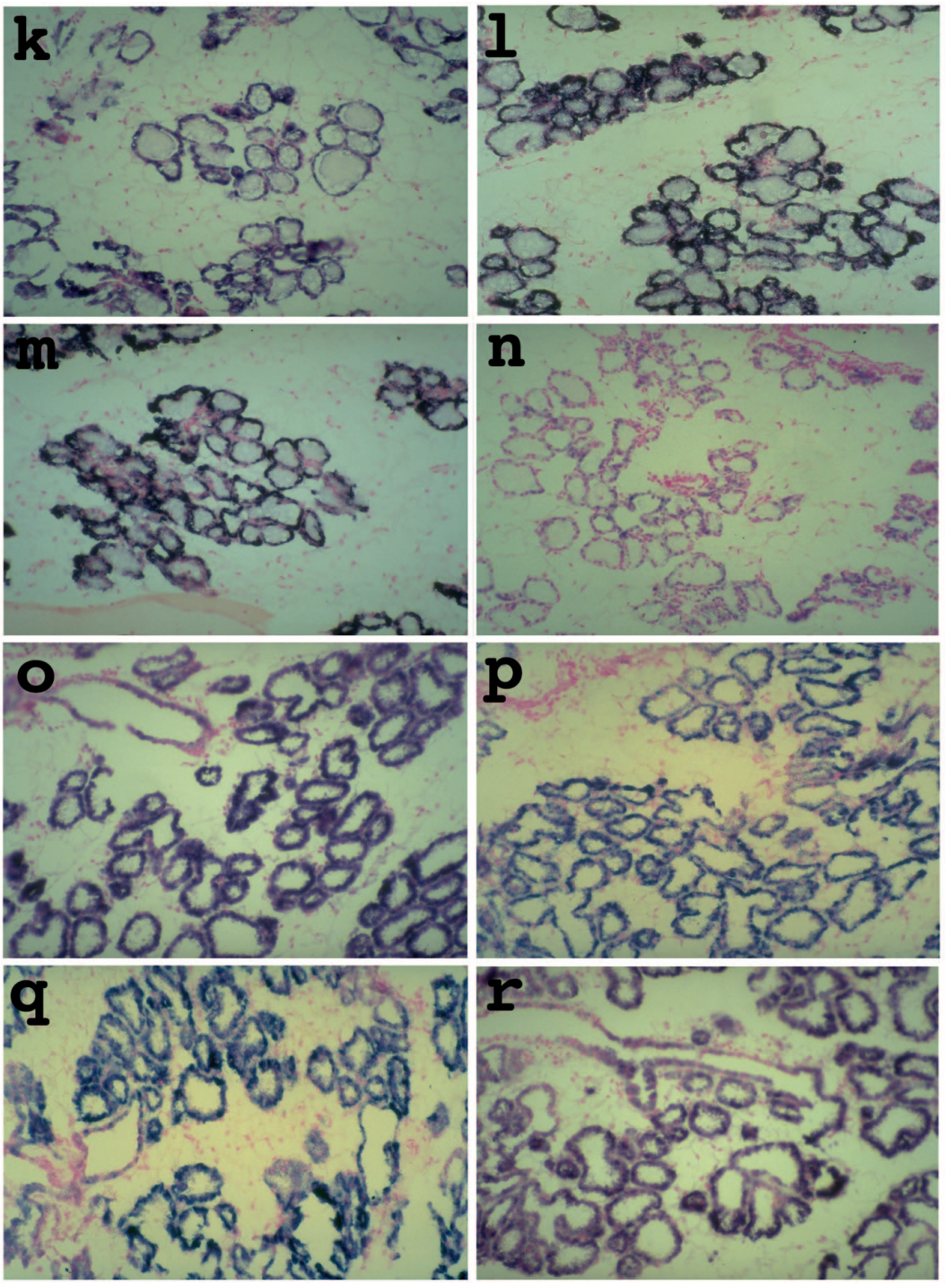

Fig. 2. In situ hybridization analysis of milk protein gene expression in non-transgenic mice. (a,b) Day 9 of pregnancy. Expression of WDNM1 (a) is seen in alveoli. Expression of β-casein (b) is sporadic. (c,d) Day 11 of pregnancy. Expression of WDNM1 (c) has increased slightly. Expression of β-casein (d) is seen in only a few alveolar cells. (e-g) Day 14 of pregnancy. (e) Widespread expression of WDNM1 in alveoli and ducts (arrow head). (f) Heterogeneous expression of β-casein in alveoli. (g) Focal expression of WAP. (h-j) Day 16 of pregnancy. (h) Strong expression of WDNM1. (i) Highly variable expression of β-casein in alveoli and low expression in ducts. (j) Uneven expression of WAP. (k-n) On day 18 of pregnancy the alveolar lumen is enlarged. Strong expression of WDNM1 (k), β-casein (l) and WAP (m). (n) Expression of α-lactalbumin is still weak. (o-r) First day of lactation. Strong expression of WDNM1 (o), very high levels of β-casein (p) and WAP (q), and increased expression of α-lactalbumin (r) are seen in the alveolar cells. Lower levels of all the milk protein genes are also observed in ductal cells. The color reaction was performed for 16 to 20 hours in all the samples, with exception of p and q where it was stopped after 2 hours. Bar, 100 μm.

cells expressed the transgene (Fig. 1E), and on day 1 of lactation bacterial β-galactosidase was found in all alveolar cells (Fig. 1F). However, even at this stage expression was not uniform, and quantitative differences were observed between individual alveolar cells.

Activation of endogenous milk protein genes

To corroborate the data obtained with the *WAP-lacZ* reporter gene, we next analyzed the activation of endogenous milk protein genes (WDNM1, β-casein, WAP and α-lactalbumin) using in situ hybridization of digoxigenin-labeled riboprobes to tissue sections. Similar to the *lacZ* reporter gene, this method permits the precise cellular localization of the detection signal. A distinct hybridization signal was obtained in mammary tissue at day 9 of pregnancy with probes for WDNM1 (Fig. 2A) and β-casein (Fig. 2B). It is evident that the expression pattern for these genes is not homogeneous. WDNM1 expression was seen in about half of the alveoli. The signal was strictly localized to alveolar cells and was not found in ductal or myoepithelial cells or in the mammary stroma. β-casein was expressed only in a few sporadic alveolar cells. On day 11 of pregnancy, alveolar development had progressed, and

WDNM1 expression was seen in most of the alveoli and in the interlobular terminal ducts (Fig. 2C). The number of alveoli that expressed β-casein had increased to approximately 30% (Fig. 2D). The same irregular distribution of β-casein transcripts was seen as before, and frequently only a few cells of one alveolus showed a strong hybridization signal. No WAP- or α-lactalbumin-specific signals were observed at these stages (data not shown).

In 14 day pregnant mice, the number of cells and the size of the alveoli had increased, and a lumen was clearly visible (Fig. 2E-G). In addition to an increase of the WDNM1 (Fig. 2E) and β-casein (Fig. 2F) signals, expression of WAP was now detected (Fig. 2G). Expression of WDNM1 was essentially uniform within alveoli, but differences in intensity occurred between alveoli. In general, a stronger hybridization signal was seen in more distal alveoli at the periphery of the gland. This regionalization of the gland has also been observed for the formation of alveolar buds (Russo et al., 1989). It appears that the most recently formed alveoli, (i.e. distal to the nipple) are more active than the more proximal older structures. Heterogeneous hybridization signals were obtained with a WAP-specific probe (Fig. 2G). WAP RNA was found sporadically and in only a few cells within any given alveolus. This pattern resembled that observed for β-casein expression at earlier stages of pregnancy (Fig. 2B,D).

Little change in the expression pattern was observed for WDNM1 between day 14 and 16 of pregnancy (Fig. 2H). The expression of β-casein RNA was slightly increased (Fig. 2I). A hybridization signal was seen already after 2-4 hours of color reaction while it took between 16 and 20 hours for a signal to develop in the earlier stages (see legend of Fig. 2). Occasional low expression of β-casein was seen also in small ducts (Fig. 2I). The signal for WAP was still uneven at this stage, but the number of WAP-expressing alveoli had increased (Fig. 2J). Analysis of closely adjacent sections showed that WAP expression was mainly found in alveoli that showed strong expression of β-casein (data not shown). The heterogeneity of milk protein expression and cellular differentiation at this stage was clearly visible at a higher magnification of sections after a short color reaction (Fig. 3A-C). Mosaic expression is particularly dramatic for the WAP gene where, in some cases, RNA was found in only 1 or 2 cells within an alveolus (Fig. 3C). The cells within one alveolus displayed great variability in the intensity of hybridization as well as in the size and number of intracellular vacuoles. It appears that the highest expression levels of any milk protein gene were found in cells with large vacuoles. This indicates a coordinated morphological and functional differentiation of the alveolar cells.

On day 18 of gestation the mammary alveoli are widely dilated, suggesting extensive secretory activity. A strong and more uniform signal for WDNM1 (Fig. 2K), β-casein (Fig. 2L) and WAP (Fig. 2M) was obtained. A much weaker signal was now also seen for α-lactalbumin (Fig. 2N).

In the lactating gland, all the alveolar secretory cells expressed the four milk proteins at a high, but still not at a uniform level (Fig. 2O-R). Lower levels of expression were also seen in ductal cells.

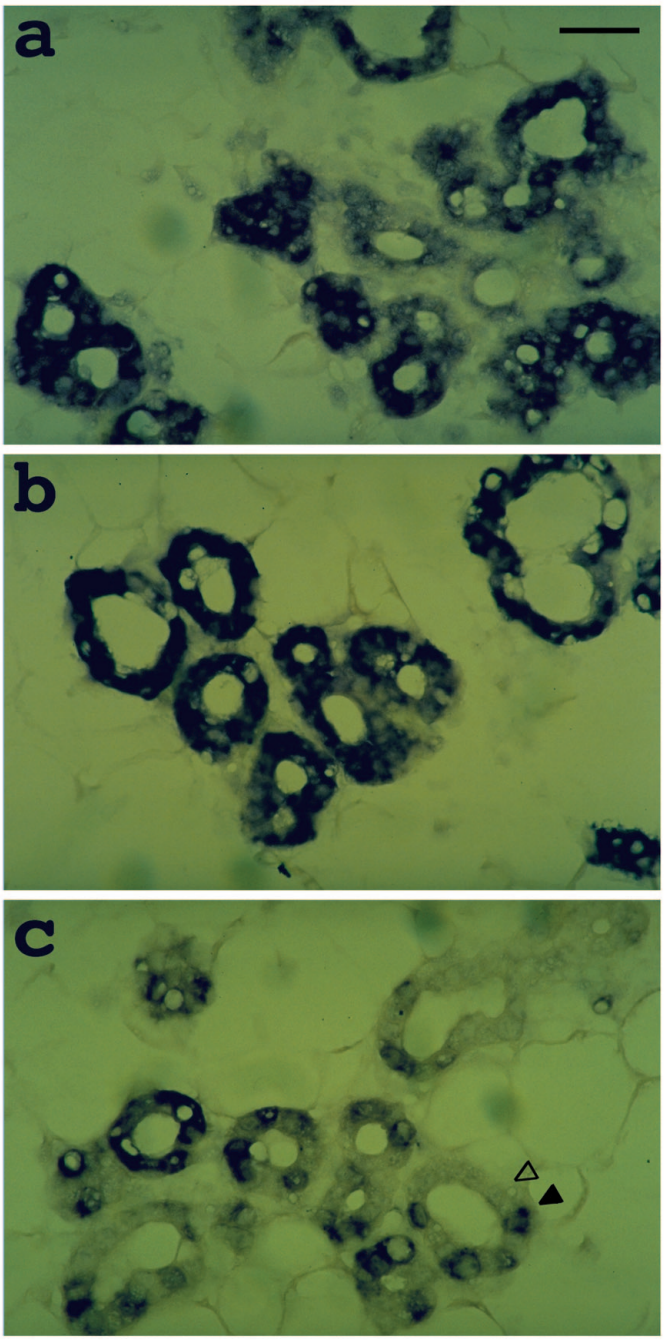

Fig. 3. Steady state levels of WDNM1 (a), β-casein (b) and WAP (c) RNA on day 16 of pregnancy. Epithelial cells within one alveolus show considerable heterogeneity in morphology and amount of milk protein expression. Note the presence of highly expressing, vacuolated cells (closed arrow head) next to cells with low expression (open arrow head). The color reaction was 4 hours. Sections were not counterstained. Bar, 25 μm.

Distribution of WAP

The heterogeneous expression of WAP transcripts seen in mid-pregnancy was compared to the distribution of WAP detectable by immunohistochemical methods (Fig. 4). On day 16 (Fig. 4A,B) and 18 (Fig. 4C,D) of pregnancy, antibodies directed against WAP showed an irregular distribution of expressing cells within individual alveoli. However, most of the immunoreactive material was concentrated in the alveolar

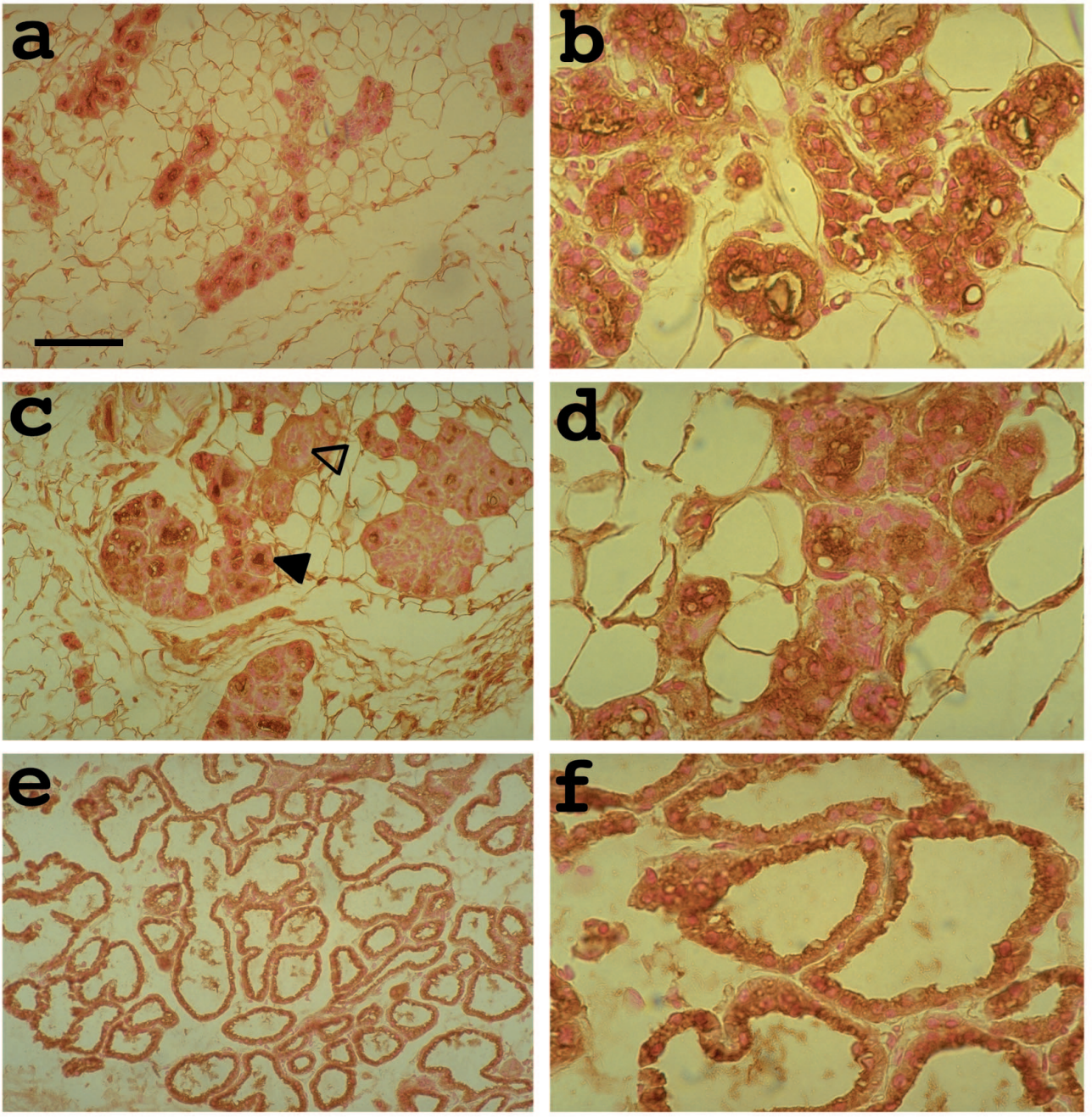

Fig. 4. Localization of WAP protein by immunohistochemistry. (a,b) On day 16 of pregnancy variable amounts of WAP protein were found in alveolar cells. (c,d) On day 18 of pregnancy increasing amounts of WAP protein were present in alveolar cells and in the lumen. The open arrow points to the lumen of an alveolus synthesizing very little WAP, and the solid arrow points to an alveolar lumen filled with WAP. (e,f) On day 1 of lactation WAP protein was present in all of the alveolar cells. Bar, 100 μm (a,c,e); 320 μm (b,d,f).

lumen. Homogeneous staining of the alveolar cells for WAP was seen during lactation (Fig. 4E,F). The absence of staining in the lumen suggests that the gland had been emptied by continuous suckling.

These results demonstrate that the mosaic expression pattern seen by in situ hybridization reflects heterogeneities in the synthetic activities of the alveolar cells and is not an artifact created by fixation and tissue processing. In addition, we saw the same distribution with frozen sections (data not shown). Lastly, we obtained differential staining patterns characteristic for a particular gene in closely adjacent sections; i.e., while WDNM1 expression in 16- and 18-day glands is uniform in many alveoli, probes for WAP and α-lactalbumin give very spotty patterns (see Fig. 3).

Milk protein gene expression in transgenic 'milchlos' mice

Experiments employing transgenic animals have shown that the deregulated expression of putative developmental genes under the control of the mouse *WAP* gene promoter can alter

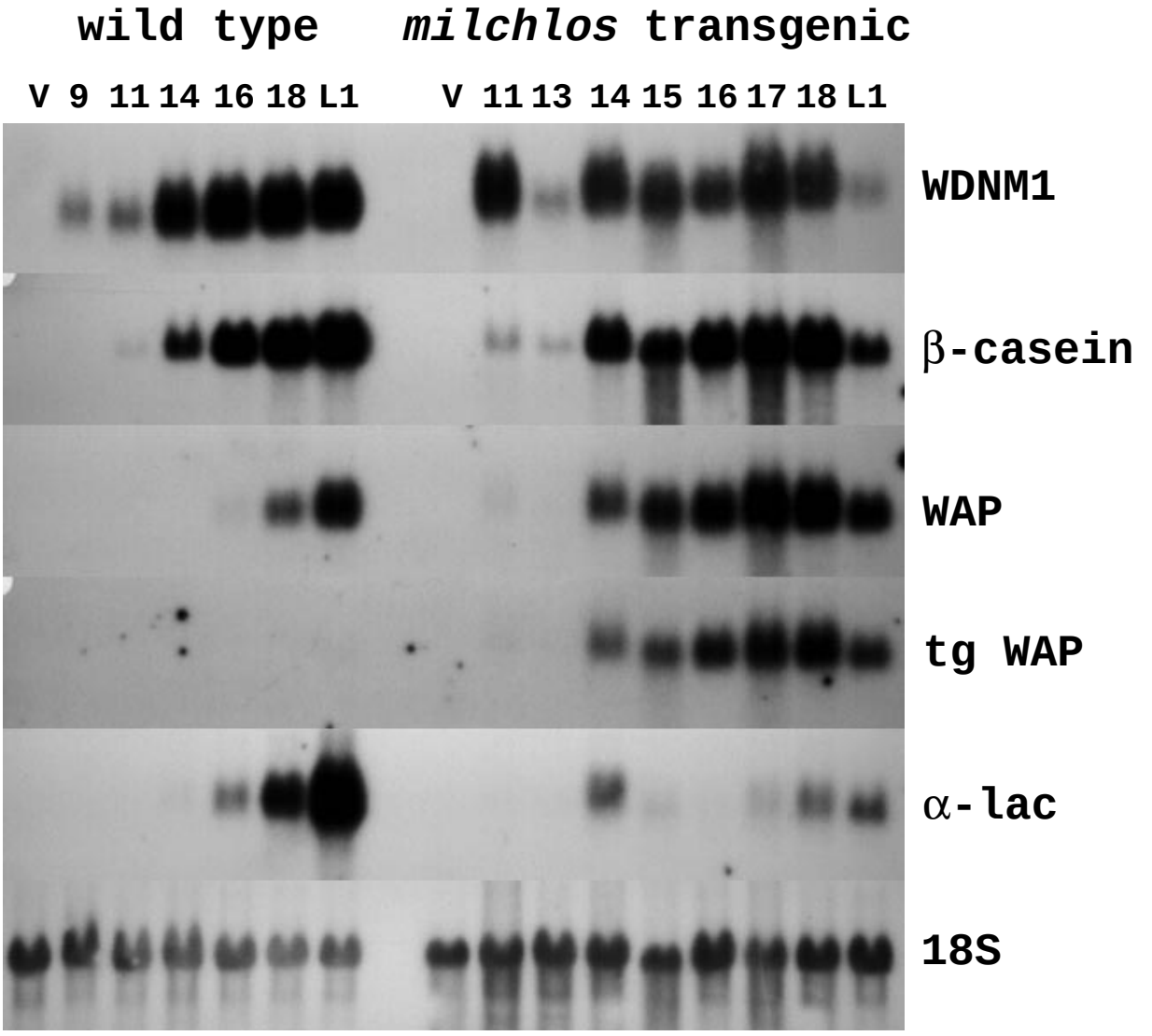

Fig. 5. Expression of milk protein genes in non-transgenic and WAP transgenic mice. 20 μg of total RNA were separated in a 1.2% formaldehyde gel, blotted and hybridized with probes specific for WDNM1, β-casein, WAP, WAP transgene, α-lactalbumin and 18S RNA. Due to a specific linker sequence introduced into the transgene (Burdon et al., 1991a), we were able to detect transgenic RNA. Although we were able to detect the transgenic RNA specifically, no specific probe was available for the endogenous WAP gene in the transgenic mice. The signal obtained with the endogenous WAP probe in transgenic mice encompasses both the endogenous and the transgenic RNA. Note, that the WAP signal obtained in transgenic mice at the early stages of pregnancy is mainly derived from the transgene.

and interfere with mammary gland development (Burdon et al., 1991b; Jhappan et al., 1993; Sympson et al., 1994). To evaluate how such changes affect cell differentiation during pregnancy, we took advantage of a line of transgenic mice that carries a mouse WAP transgene (Burdon et al., 1991b). Female mice from this line cannot lactate (the 'milchlos' phenotype) and exhibit incomplete mammary gland development during pregnancy. This defect appears to be correlated with a precocious expression of the transgene (Burdon et al., 1991b). In the original description of the 'milchlos' mouse, an incomplete penetration of the agalactic phenotype was observed. A wide range of histological variability was also seen during all stages of mammary development (Fig. 6D-I). It is possible that the histological variability results from mosaic expression of the transgene comparable to the activity of endogenous genes. We, therefore, analyzed the expression patterns of the WAP transgene and of endogenous milk protein genes by RNA blotting (Fig. 5) and by in situ hybridization (Fig. 6).

In Fig. 5, we compared the expression of milk protein genes in non-transgenic and WAP transgenic animals. In non-transgenic mice, only WDNM1 was expressed at significant levels on day 9 of gestation. The steady state level of this RNA peaked around day 14 of pregnancy, and no further increase was observed at later stages. β-casein RNA was detected on day 11 of pregnancy and its amount increased throughout pregnancy. A WAP signal was seen on day 16 of pregnancy, followed by at least a 30-fold induction on day 1 of lactation. The induction pattern of α-lactalbumin followed that of WAP, but at a lower level (note that the exposure time for the α-lactalbumin RNA in Fig. 5 was approximately 10-fold longer than

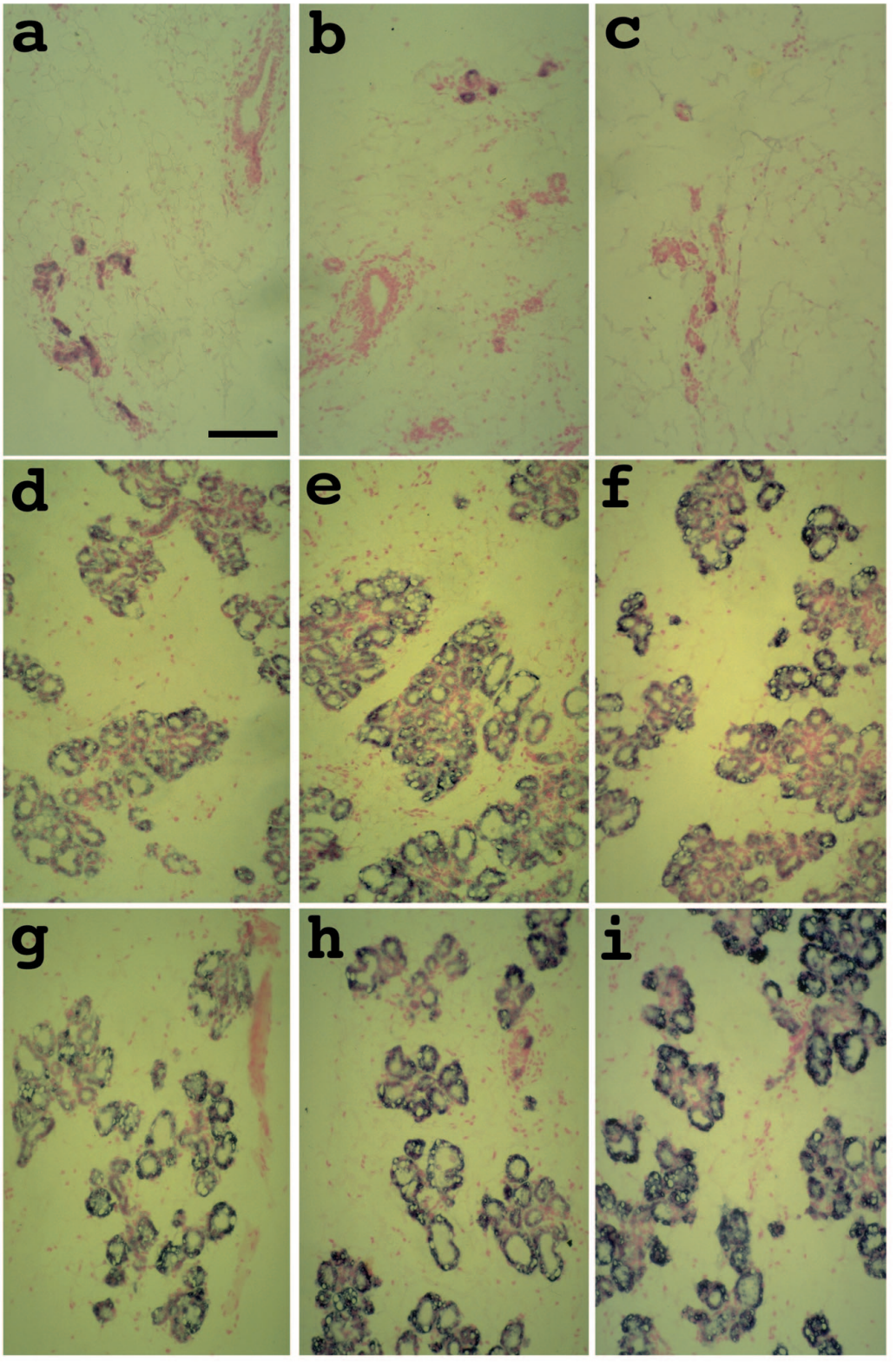

Fig. 6. Expression of milk proteins in WAP transgenic mice. (a-c) On day 9 of pregnancy WDNM1 (a), β-casein (b) and WAP (c) are sporadically expressed. (d-f) On day 16 of pregnancy the alveoli display an altered morphology compared to non-transgenic mice. Great variations in the levels of milk protein expression are observed. WDNM1 (d), β-casein (e), WAP (f). (g-i) morphological differentiation and expression of milk proteins appear arrested on day 18 of pregnancy. WDNM1(g), β-casein (h), WAP (i). It should be noted that the mammary gland in the animal at day 18 of pregnancy is less developed than the one at 16 days. This reflects the much greater animal to animal variation observed in the transgenics. Color reactions were performed 1 hour (f), 2 hours (g,h), 3 hours (d,e) and overnight (a-c). Bar, 100 μm.

that for the other milk protein genes). After a long exposure, low signals for all four genes were seen in virgin animals (not shown).

The expression of milk proteins in the WAP/WAP-transgenic mice was much more variable than in non-transgenic littermates (Fig. 5). This was particularly emphasized with the *WDNM1* and *α-lactalbumin* genes. Precocious expression of the *α-lactalbumin* gene was see on day 14 of pregnancy. However, the level of RNA at late pregnancy and at parturition was only 5% of that found in non-transgenic mice. Furthermore, expression of the *WAP*, *β-casein* and *WDNM1* genes remained steady throughout the second part of pregnancy and

declined at parturition. This decline was particularly steep in the case of WDNM1. A possible explanation for the sharp decrease of WDNM1 would be the inability of alveolar cells to maintain a differentiation stage compatible with WDNM1 expression. The fact that α-lactalbumin RNA levels never increase during pregnancy further suggests that the cells never progress into the status of terminal differentiation.

By in situ hybridization, WAP RNA was seen on day 9 of pregnancy (Fig. 6C). As in the non-transgenic mice, when the milk protein genes are first expressed, transcripts are seen in only a few cells. This signal most likely comes from the transgenic RNA (see legend to Fig. 5). At day 9 of pregnancy, the expression level and pattern of WDNM1 (Fig. 6A) and β-casein (Fig. 6B) were similar to those in non-transgenic animals. On day 11 to 14, WAP was expressed in more alveoli but in the same irregular pattern as before. The hybridization signal for WDNM1 and β-casein was reduced compared to the non-transgenic animals (data not shown). In addition, from day 16 of pregnancy onwards, we observed distinct morphological differences between non-transgenic and transgenic animals. The alveoli of the transgenic mice appeared much more compact. The epithelial cells retained a cuboidal shape and contained more vacuoles (Fig. 6D-I; compared to non-transgenic animals in Fig. 2H-N). At these stages, the expression of WDNM1 (Fig. 6D,G) and β-casein was reduced (Fig. 6E,H), while the expression of WAP had increased (Fig. 6F,I). A weak signal was also obtained for α-lactalbumin (data not shown). It is important to emphasize that the expression pattern for all the milk protein genes remained much more heterogeneous. The alveoli never enlarged and the alveolar cells maintained their cuboidal shape. This clearly reflects the inability of these mammary glands to reach the fully differentiated state, which becomes evident in the inability of the WAP transgenic mice to nurse their pups. Toward the end of pregnancy and immediately after parturition, we saw an increase in apoptotic alveolar cells compared to non-transgenic animals (data not shown). It is possible that the cells exhibiting a deregulated expression of WAP and α-lactalbumin are eliminated and a loss of cells may contribute to the lack of alveolar development.

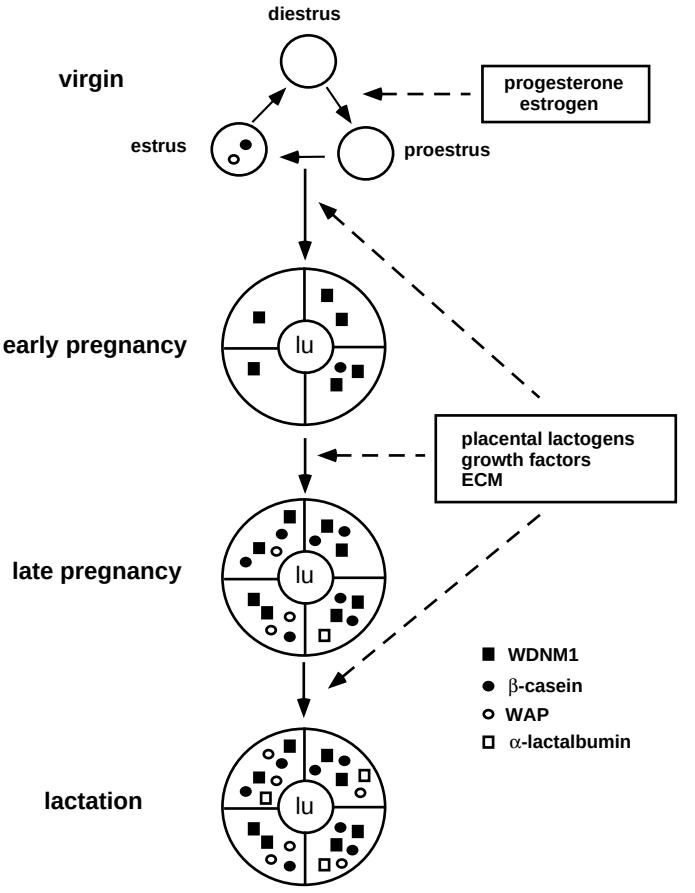

Fig. 7. Model for the regulation of differentiation of mammary alveolar cells during puberty, pregnancy and lactation. Ovarian steroid hormones induce transient expression of milk protein genes during the estrus period of the cycle. Terminal differentiation is achieved during pregnancy when placental lactogens induce the sequential activation of the *WDNM1*, *β-casein*, WAP and α-*lactalbumin* genes. Localized growth factors and the extracellular matrix (ECM) are probably involved in mediating the effects of the lactogenic hormones. Mammary alveolar cells in the virgin gland are presented as circles. During pregnancy and lactation they are presented as part of an alveolar structure surrounding a lumen (lu).

DISCUSSION

We have demonstrated that mammary epithelial cells in virgin mice go through a transient differentiation process: expression of milk protein genes occurs during estrus and then abates. Full differentiation is established and maintained only during pregnancy, and finally results in a nearly homogenous population of secretory cells. During pregnancy, the expression of individual genes varies greatly between neighboring cells within alveoli. This suggests that the onset and progression of differentiation occurs asynchronously among individual cells. Based on these results, we propose two distinct phases in mammary alveolar epithelial development (Fig. 7). First, a transient differentiation phase which is induced by ovarian hormones in the cycling virgin, and secondly, the establishment of terminal differentiation during pregnancy. The asynchronous development of alveolar cells during pregnancy indicates that the differentiation status of individual alveolar cells, as determined by the presence of systemic hormones, is modulated by local factors. Further asynchrony may result from temporal differences in cell proliferation events which give rise to terminally differentiated cells in the G_0 phase of the cell cycle.

Transient and asynchronous alveolar differentiation

The *WAP* gene promoter is active in approximately 30% of ductal and alveolar cells of virgin mice in estrus but not in diestrus or proestrus. The fate of these differentiated cells is not clear. They may cease expressing milk protein genes because of a change in hormonal signaling, but may persist and subsequently progress through pregnancy where they would resume a permanently differentiated state. Alternatively, these cells may die after having undergone differentiation. Evidence that differentiated cells persist in the virgin gland comes from transgenic mice that express growth modulators, such as the SV40T-antigen (Tzeng et al., 1993), TGFα (Eric Sandgren et al., unpublished data), TGFβ1 (Jhappan et al., 1993; Kordon et al., 1995;), a WAP transgene (Burdon et al., 1991b) or

stromelysin (Sympson et al., 1994), under control of the *WAP* gene promoter. In these mice, phenotypic changes can be seen already in mature virgins.

The developmental and molecular basis of asynchronous cell differentiation

The differentiation of mammary epithelial cells, and the concomitant expression of milk protein genes, is controlled by a network of regulatory signals. Two processes are required to produce a differentiated cell from its predetermined precursor. A program to cause the cell to be determined and a subsequent environment that allows the cell to differentiate along its predetermined course. During organ development, a predetermined stem cell produces two daughters. One remains a stem cell, the other differentiates. However, differentiating cells are capable of additional rounds of proliferation before eventually giving rise to terminally differentiated cells. The sequential expression of milk proteins in the proliferating and differentiating mammary epithelium is indicative of this process. The delay in onset of expression of α-lactalbumin and WAP in the majority of the differentiating secretory cell population suggests that these genes are expressed late in the differentiative pathway. In fact, their expression may be linked to terminal differentiation in the sense of the loss of proliferative capacity, i.e. entering a state of G_0. We propose that precocious expression of WAP results in terminal differentiation of alveolar cells already at mid-pregnancy, which in turn prevents the alveolar structures from proliferation.

The primary stimuli of mammary gland development during pregnancy are provided by progesterone, estrogen and lactogenic hormones. In addition, these hormones participate in the control of milk protein gene expression. The regulatory features imposed by these hormones vary between different milk protein genes. For example, the induction of *WAP* gene expression requires a synergistic activity of prolactin and glucocorticoids, but the *β-casein* gene is transcribed in the presence of prolactin alone (Hennighausen et al., 1988; Burdon et al., 1991a; McKnight et al., 1991; Shamay et al., 1992). Although response elements for the two signalling pathways have been identified in the promoter regions of both genes (Li and Rosen, 1994, 1995), their mode of action in the differential gene induction is not understood. Additional signals, possibly of autocrine or paracrine nature, are required to achieve the temporal coordination of mammary secretory development, differentiation and gene expression. This is supported by experiments with transgenic mice in which the expression of growth modulators is deregulated. TGFβ1 (Jhappan et al., 1993; Pierce et al., 1993; Kordon et al., 1995), stromelysin (Sympson et al., 1994), and WAP (Burdon et al., 1991b; Hennighausen et al., 1994) have been shown to influence mammary gland development and differentiation in vivo. Depending on the growth factor used and the temporal onset of expression, different phenotypes were observed. This suggests that these molecules intersect at different stages in the differentiation process (Robinson et al., unpublished data). TGFβ1 and WAP elicit their effect only over a very short range, and probably act through an intracrine or autocrine mechanism (Kordon et al., 1995; Hennighausen et al., 1994).

Phenotypic penetrance of altered mammary differentiation

The phenotypic changes in ductal and alveolar development, induced by the deregulated expression of a particular growth regulator, can vary dramatically between individual animals. The penetrance of a phenotype is not only dependent on the site of integration, but likely also on the heterogenous and asynchronous expression imposed by the promoter in the transgene. As shown here in mice transgenic for the mouse *WAP* gene, the expression pattern of differentiation markers can vary between individual mice. Although the overall differentiation and development of mammary tissue in these mice is inhibited and results in the same *milchlos* phenotype, variations in the state of alveolar differentiation are observed between animals. Common to all transgenic mice, however, is the precocious expression of the *WAP* transgene and the inability of alveolar cells to enter a state of differentiation which permits full activation of the *α-lactalbumin* gene. This deregulated activation of growth regulators has been used successfully to study mechanisms underlying mammary development and differentiation. However, a full utilization of this approach requires a temporal control of transgene activation. Further experiments employing precise temporal regulation of gene expression using an inducible system such as the tetracycline responsive system (Furth et al., 1994; Hennighausen et al., 1995) will define more clearly the role of the extracellular matrix, growth factors and their receptors in the regulation of mammary gland development and lactation.

REFERENCES

Burdon, T., Sankaran, L., Wall, R. J., Spencer, M. and Hennighausen, L. (1991a). Expression of a whey acidic protein transgene during mammary development: Evidence for different mechanisms of regulation during pregnancy and lactation. *J. Biol. Chem.* **266**, 6909-6914.

Burdon, T., Wall, R. J., Shamay, A., Smith, G. H. and Hennighausen, L. (1991b). Over-expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype. *Mech. Devel.* **36**, 67-74.

Chen, L.-H. and Bissell, M. J. (1989). A novel regulatory mechanism for whey acidic protein gene expression. *Cell Regul.* **1**, 45-54.

Chomczynski, P. and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Daniel, C. W. and Silberstein, G. B. (1987). Postnatal development of the rodent mammary gland. In *The mammary gland: development, regulation and function* (eds. M. C. Neville, C. W. Daniel), pp. 3-36. New York: Plenum Press.

Forsyth, I. A. (1986). Variation among species in the endocrine control of mammary growth and function: the roles of prolactin, growth hormone and placental lactogen. *J. Dairy Sci.* **69**, 886-903.

Furth, P. A., St. Onge, L., Boger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. and Hennighausen, L. (1994). Temporal control of gene expression in transgenic mice by a tetracycline responsive promoter. *Proc. Acad. Natl. Sci. USA* **91**, 9302-9306.

Hennighausen, L., Westphal, C., Sankaran, L. and Pittius, C. W. (1988). Regulation of expression of milk protein genes. In Transgenic *Technology in Medicine and Agriculture* (eds. N. First and F. Haseltine), pp. 61-70. Boston: Butterworth.

Hennighausen, L., Wall, R. J., Tillmann, U., Li, M. and Furth, P. A. (1995) Conditional gene expression in secretory tissues and skin of transgenic mice using the MMTV-LTR and the *tetracycline* responsive system, *J. Cell Biochem.* (in press).

Hennighausen, L., McKnight, R. A., Burdon, T., Baik, M., Wall, R. J. and Smith, G. H. (1994). Whey acidic protein extrinsically expressed from the mouse mammary tumor virus long terminal repeat results in hyperplasia of the coagulation gland epithelium and impaired mammary development. *Cell Growth Diff.* **5**, 607-613.

Jhappan, C., Geiser, A. G., Kordon, E. C., Bagheri, D., Hennighausen, L., Roberts, A. B., Smith, G. H. and Merlino, G. (1993). Targeting expression

of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J.* **12**, 1835-1845.

Joseph, R. and Collet, C. (1994). Double staining *in situ* study of mRNAs encoding milk proteins in the mammary gland of the tammar wallaby (*Macropus eugenii*). *J. Reprod. Fertil.* **101**, 241-246.

Kordon, E., McKnight, R. A., Jhappan, C., Hennighausen, L., Merlino, G. and Smith, G. H. (1995). Ectopic TGFβ1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev. Biol.* **167**, 47-61.

Li, S. and Rosen, J. M. (1994). Glucocorticoid regulation of rat whey acidic protein gene expression involves hormone-induced alterations of chromatin structure in the distal promoter region. *Mol. Endocrinol.* **8**, 1328-1335.

Li, S. and Rosen, J. M. (1995). CTF/NF1 and mammary gland factor (STAT5) play a critical role in regulating rat whey acidic protein gene expression in transgenic mice. *Mol. Cell. Biol.* **15**, 2063-2070.

Lin, C. Q. and Bissell, M. J. (1993). Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J.* **7**, 737-743.

McKnight, R. A., Burdon, T., Pursel, V. G., Shamay, A., Wall, R. J. and Hennighausen, L. (1991). The Whey Acidic Protein. In *Breast Cancer: Molecular and Cellular Biology* (eds. Dickson and Lippman), pp. 399-412. Boston: Kluwer Academic Publishers.

Mills, E. S. and Topper, Y. J. (1970). Some ultrastructural effects of insulin, hydrocortisone, and prolactin on mammary gland explants. *J. Cell Biol.* **44**, 310-328.

Molenaar, A. J., Davis, S. R. and Wilkins, R. J. (1992). Expression of α-lactalbumin, α-S1-casein, and lactoferrin genes is heterogenous in sheep and cattle mammary tissue. *J. Histochem. Cytochem.* **40**, 611-618.

Morrison, B. and Leder, P. (1994). Neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2 initiated tumors. *Oncogene* **9**, 3417-3426.

Ormerod, E. J. and Rudland, P. S. (1984). Cellular composition and organization of ductal buds in developing rat mammary glands: evidence for morphological intermediates between epithelial and myoepithelial cells. *Am. J. Anatomy* **170**, 631-652.

Paleyanda, R. K., Zhang, D.-W., Hennighausen, L., McKnight, R. A. and Lubon, H. (1994). Regulation of human protein C gene expression by the mouse WAP promoter. *Transgenic Research* **3**, 335-343.

Pierce, D. F. Jr., Johnson, M. D., Matsui, Y., Robinson, S. D., Gold, L. I., Purchio, A. F., Daniel, C. W., Hogan, B. L. M., Moses, H. L. (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1. *Genes Dev.* **7**, 2308-2317.

Pitelka, D. R., Hamamoto, S. T., Duafala, J. G. and Nemanic, M. K. (1973). Cell contacts in the mouse mammary gland. *J. Cell Biol.* **56**, 797-818.

Pittius, C. W., Sankaran, S., Topper, Y. and Hennighausen, L. (1988). Comparison of the regulation of the whey acidic protein gene to a hybrid gene containing the whey acidic protein gene promoter in transgenic mice. *Mol. Endocrinol.* **2**, 1027-1032.

Rudland, P. S. and Hughes, C. M. (1989). Immunocytochemical identification of cell types in human mammary gland: variations in cellular markers are dependent on glandular topography and differentiation. *J. Histochem. Cytochem.* **37**, 1087-1100.

Russo, I. H., Tewari, M. and Russo, J. (1989). Morphology and development of the rat mammary gland. In *Integument and Mammary Glands* (eds. T. C. Jones, V. Mohr and R. D. Hunt), pp. 233-252. Berlin, Heidelberg: Springer-Verlag.

Shamay, A., Pursel, V. G., McKnight, R. A., Alexander, L., Beattie, C., Hennighausen, L. and Wall, R. J. (1991). Production of the mouse whey acidic protein in transgenic pigs during lactation. *Journal of Animal Science* **69**, 4552-4562.

Shamay, A., Pursel, V. G., Wall, R. J. and Hennighausen, L. (1992). Induction of lactogenesis in transgenic virgin pigs: Evidence for gene and integration site-specific hormonal regulation. *Mol. Endocrinol.* **6**, 191-197.

Smith, G. H., Mehrel, T., Roop, D. R. (1990). Differential keratin gene expression in developing, preneoplastic, and neoplastic mouse mammary epithelium. *Cell Growth Diff.* **1**,161-170.

Streuli, C. H., Bailey, N. and Bissell, M. J. (1991). Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *J. Cell Biol.* **115**, 1383-1395.

Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J. and Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J. Cell Biol.* **125**, 681-693.

Topper, Y. J. and Freeman, C. S. (1980). Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol. Rev.* **60**, 1049-1056.

Tzeng, Y.-J., Guhl, E., Graessmann, M. and Graessmann, A. (1993). Breast cancer formation in transgenic animals induced by the whey acidic protein SV40 T antigen (WAP-SV-T) hybrid gene. *Oncogene* **8**, 1965-1971.

Wakao, H., Gouilleux, F. and Groner, B. (1994). Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.* **13**, 2182-2191.

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